Treatment of Viral Infections With 5-Aminolevulinic Acid and Light

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Background and Objective: **When 5-aminolevulinic acid (ALA) is exogenously supplied, protoporphyrin IX (PpIX) is accumulated in various cells and makes them light sensitive. The possibility of using such an approach for the treatment of viral infections was studied in this work.**
Study Design/Materials and Methods: **ALA was added to cultured cells infected with human immunodeficiency virus (HIV). Accumulation of PpIX in the cells as well as virus infectivity after photodynamic treatment (PDT) were assessed. For in vivo studies, guinea pigs were infected with herpes simplex virus (HSV) and then administered ALA at intervals after infection. The animals were exposed to PDT at the site of infection 3 hours after ALA administration. Clinical observations and virus titration were made daily. For clinical studies, two patients with *Molluscum contagiosum* and *Verrucae vulgares* were treated with ALA fortified with an iron chelating agent and dimethylsulfoxide, followed 4 hours later by PDT.**
Results: **Cells that are infected with HIV accumulated PpIX upon addition of ALA in vitro. This accumulation was enhanced ~two-fold in the presence of an iron chelator. Subsequent exposure to red light PDT drastically reduced the virus titer (> 99% for U1 cells latently infected with HIV). In guinea pigs infected with HSV, subsequent administration of ALA and exposure of the lesions to red light shortened the duration of vesicles' appearance from more than a week to a few days and reduced HSV titer in the lesions by $\geqslant 5 \log_{10}$. ALA-PDT treated AIDS patient suffering from *Molluscum cotagiosum* or a kidney transplant patient with *Verrucae vulgares* showed greatly improved clinical symptoms one month after treatment.**
Conclusion: **It is concluded that ALA-PDT could be effective in treating certain viral infections, particularly those resulting in warts. Lasers Surg. Med. 21:351–358, 1997.** **© 1997 Wiley-Liss, Inc.**

Key words: **virus inactivation; photodynamic treatment; human immunodeficiency virus; herpes simplex virus; warts**

INTRODUCTION

Photodynamic therapy (PDT) mediated by 5-aminolevulinic acid (ALA) was proposed in 1990 as a new anticancer treatment [1]. Indeed, topical application of ALA followed by exposure to light has been used successfully for eradication of vari-

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ous skin cancers in clinical studies [2–4]. In addition, ALA-PDT has been suggested for treating mycosis fungoides [5] and ablation of the endometrium as an alternative to hysterectomy or for sterilization [6]. The potential of topical ALA-PDT for treatment of plaque psoriasis has also been demonstrated [7,8].

ALA is a precursor of haem biosynthesis and its synthesis is rate-limiting in the pathway. When ALA is supplied exogenously protoporphyrin IX (PpIX) is accumulated because conversion of PpIX to haem by ferrochelatase becomes rate-limiting [9]. Because PpIX is a photosensitizer, subsequent exposure to light leads to cell destruction, primarily by damage to the mitochondria [10].

In this paper we show that ALA-PDT is an effective way to inactivate intracellular viruses such as herpes simplex virus (HSV) and human immunodeficiency virus (HIV) both in vitro and in vivo. It is further shown that topical ALA-PDT augmented with an iron chelator and dimethylsulfoxide (DMSO) can be used to treat viral infections resulting in warts, such as *Verrucae vulgares* or *Molluscum contagiosum*, which currently lack effective treatments. The former is caused by a papilloma virus and the latter by Molluscum contagiosum virus (MCV), a human pox virus that is responsible for an extensive and essentially untreatable opportunistic disease that frequently occurs in individuals with AIDS [11].

MATERIALS AND METHODS

Cell Lines

U1 cells that are latently infected with HIV [12] were obtained from Dr. Thomas Folks, National Institutes of Health, and cultured as above. Following treatment, the cells were induced to express HIV by addition of 100 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St. Louis, MO) for 24 hours. The cultures were then rinsed twice and production of HIV was measured by focal immunoassay [13].

TABLE 1. Kinetics of PpIX Accumulation in U1 Cells*

Incubation time (hour)	PpIX in 10^6 cells (pmole)
0	0.2 ± 0.2
1	2.5 ± 0.4
3	12.2 ± 0.7
5	17.0 ± 1.0
7	10.1 ± 0.8

*PpIX was determined as described in Materials and Methods following incubation of the cells with 1 mM ALA for the indicated times. Data are the mean of triplicates ± SEM.

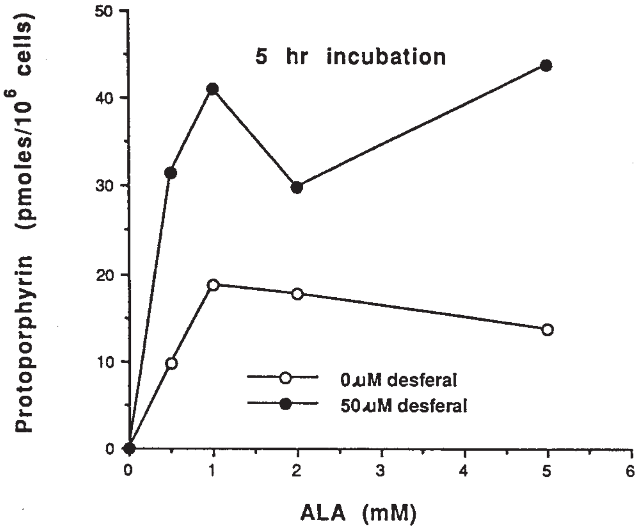

Fig. 1. Induction of PpIX accumulation in U1 cells. Cells were incubated with ALA in the growth medium at the indicated concentration for 5 hours with (●) or without (○) 50 µM desferal. The cells were then extracted with 5% HCl and PpIX content was measured using spectrofluorimetry, as described in Materials and Methods.

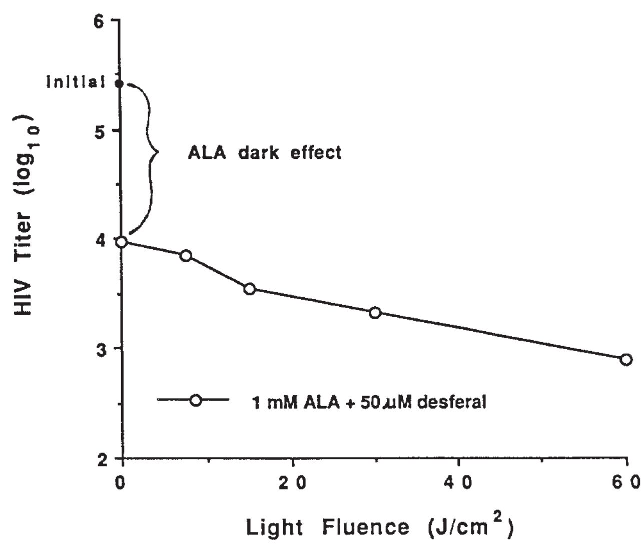

Fig. 2. HIV inactivation in U1 cells grown with ALA and exposed to red light. Cells were incubated with 1 mM ALA and 50 µM desferal for 5 hours and then exposed to graded light doses. HIV titer was assayed as described in Materials and Methods.

Animals

Guinea pigs (Hartley) were used at 200–300 g, both males and females. In each experiment there were five to ten animals.

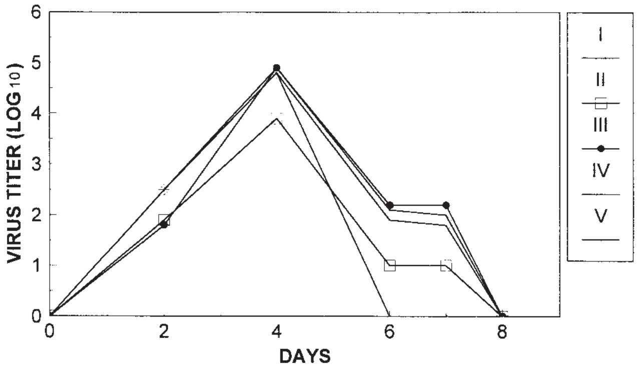

Fig. 3. HSV titration in infected guinea pigs. HSV was titrated as described in Materials and Methods at different days after infection. The different symbols represent different animals.

Virus Isolation In Vivo

Specimens for viral cultures were taken from lesions on the backs of guinea pigs by gently rubbing a cotton-tipped swab over the lesions and placing them into 2 ml of transport medium. The specimens were transferred immediately to the laboratory. For viral culture the swabs were agitated and inoculated into tubes containing monolayers of human embryo fibroblasts or green monkey kidney cells (Vero), pretreated with 10 μM dexamethasone. After incubation at 37°C for 24 hours the medium was replaced with fresh Eagle's minimal essential medium supplemented with nonessential amino acids (MEM-NAA) containing 2% fetal calf serum. The cultures were examined for cytopathic effect (CPE). When the cells showed 50% CPE they were harvested, washed and used to prepare slides for immunofluorescent staining for HSV confirmation.

Light Exposure

The light source was a 500 W xenon short arc lamp (Versa Light, Medic Lightech, Ltd., Haifa, Israel) filtered to isolate a wide band red light (590–700 nm) and transmitted via a fiber bundle at an irradiance of 100 mW/cm^2. The light dose delivered was 18 J/cm^2 for guinea pigs and 120 J/cm^2 in the clinical setting.

Chemicals and Application

ALA was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in phosphate buffered saline and added to cells at 1 mM final concentration, unless indicated otherwise. For animal experiments ALA was administered intraperitoneally at 240 mg/kg body weight, at various times

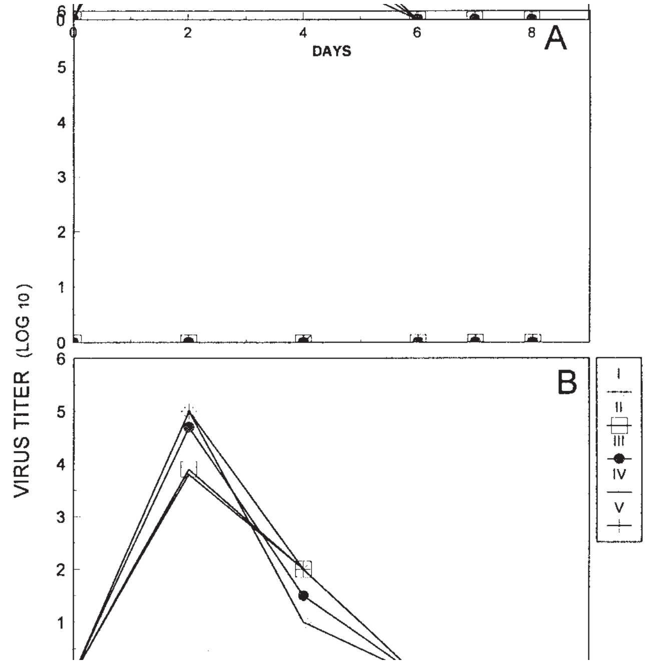

Fig. 4. Guinea pigs infected with HSV and shortly thereafter administered ALA. 3 hours later the infected area was exposed to 18 J/cm^2 of red light **(A). B:** Animals which served as a dark control were administered ALA as above. Virus titration was as described in Materials and Methods.

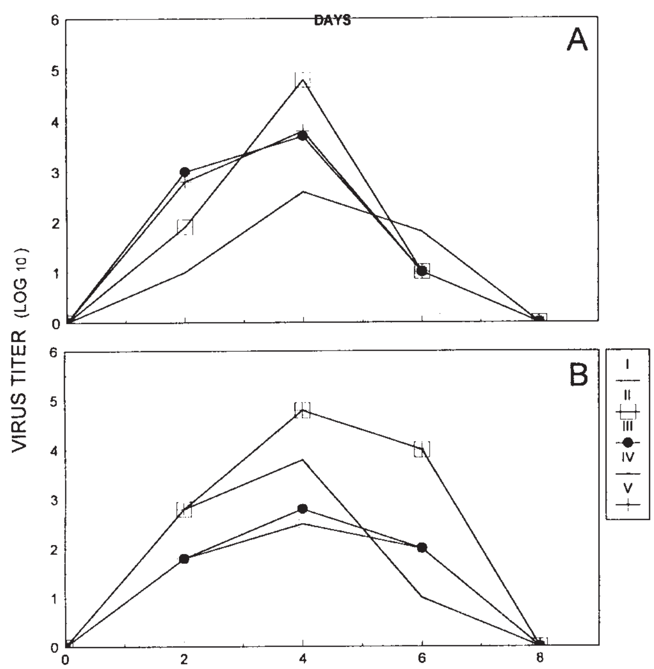

Fig. 5. Guinea pigs infected with HSV and treated with ALA-PDT 2 days later. For details see legend to Figure 4.

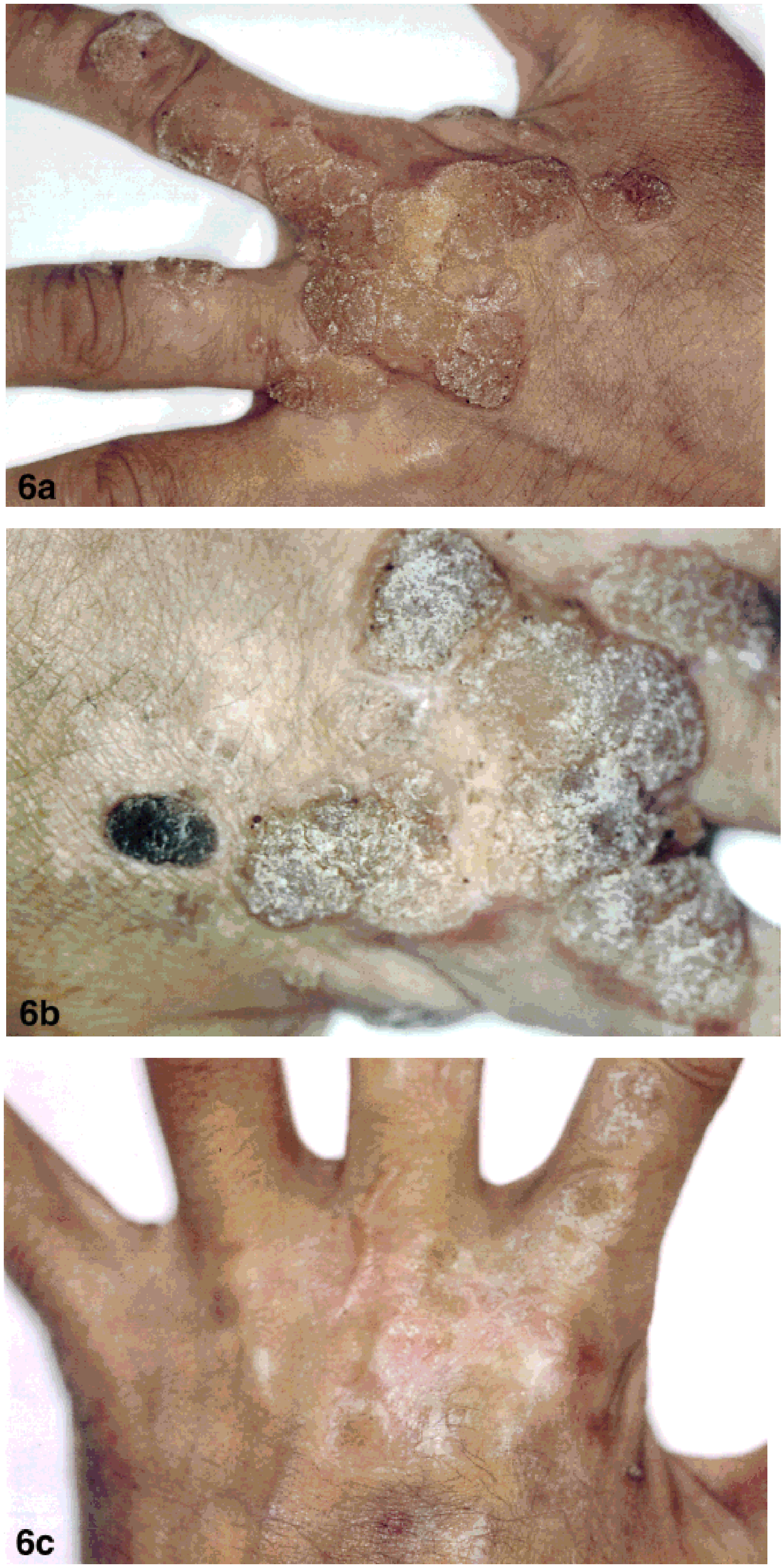

Fig. 6. Case 1. **A:** A massive *verrucae vulgares* of hands. **B:** 7 days after ALA-PDT (crust formation). **C:** 1 month after treatment.

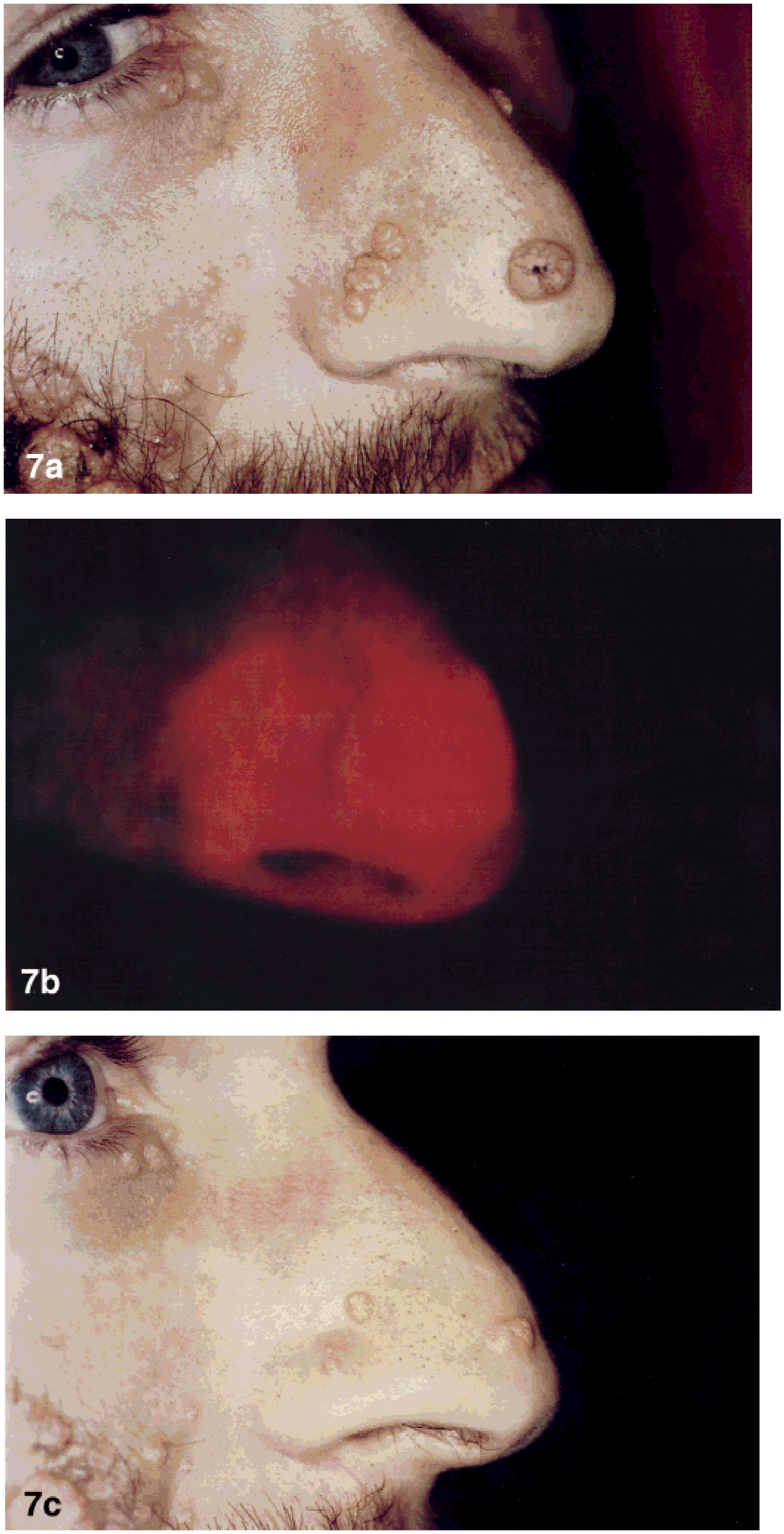

Fig. 7. Case 2: **A:** *Molluscum contagiosum* in an AIDS patient. **B:** Fluorescence of PpIX, 4 hours after application of ALA. **C:** 1 month after treatment.

after infection with HSV. Animals were exposed to light 3 hours after ALA administration. For treatment of humans, 20% ALA cream containing 2% DMSO and 2% ethylenediamine tetraacetic acid (EDTA) was prepared prior to application and applied to the lesion after cleaning the area with a saline solution (0.2 ml of cream to 1 cm^2 of skin area). After application, the skin was covered by a plastic adhesive dressing and an aluminum foil shield for protection from light exposure. The cream was left on the skin 4–5 hours. Prior to light exposure the cream was removed.

PpIX Fluorescence Measurements

To observe the production of PpIX in the lesion after ALA application, the red fluorescence of PpIX was photographed during illumination of the area with a medical-surgery UV examining light source (Burton Division, Cavitron Corp., Van Nuys, CA). PpIX in U1 cells was measured following extraction of 5×10^6 cells in 3 ml of 5% HCl for 30 minutes at 4°C. Prior to extraction the cells were washed twice in PBS. PpIX in the cell extract was determined fluorometrically with a Shimadzu spectrofluorimeter (model RF-1501), using PpIX as a reference standard. HPLC determinations established that more than 90% of the fluorescence was associated with PpIX.

RESULTS

In Vitro

In order for ALA-PDT to be effective, ALA concentration and time of incubation have to be optimized to obtain maximal accumulation of PpIX in the cells. In most cases it takes 3–6 hours to reach the peak of PpIX accumulation. Table 1 shows that in U1 cells latently infected with HIV, this peak occurred 5 hours after ALA was added to the growth medium. This accumulation was maximal at 1 mM ALA and was further enhanced ~two-fold when desferal was also added (Fig. 1). This enhancement is consistent with previous observations [8] and is due to chelation of iron by desferal, thereby inhibiting conversion of PpIX to haem by ferrochelatase. For subsequent experiments we used these conditions (1 mM ALA, 50 μM desferal and 5 hour incubation) to maximize the virucidal effect. Figure 2 shows that under these conditions about 1.5 $\log_{10}$ inactivation of HIV occurs in U1 cells in the dark. Exposure to light progressively inactivated HIV as a function of light dose, reducing HIV titer by 2.6 $\log_{10}$ at 60 J/cm^2.

In Vivo

Clinical observations. Inoculation of HSV on the backs of guinea pigs resulted in a local infection starting after 24 hours, manifested as reddening and swelling for up to 3 days. At 3–6 days vesicles were formed, followed by the appearance of crusts during the second week. Complete healing occurred at 3–4 weeks after infection. Exposure to light or ALA only at various times after infection had no obvious effect on the clinical manifestations. When treated with ALA-PDT immediately or up to 6 hours after infection there was a dramatic effect. Duration of vesicles' appearance was very short and healing started on the third day. Crusting time, however, was longer and the diameter of the crusts was 2 cm instead of 0.3–0.5 cm in the controls. The crusts remained for about a month and the irradiated area remained hairless for 6 weeks. ALA-PDT administered 24 hours or longer after infection had no effect on the manifested signs.

Virus titration. The clinical observations were confirmed by titrating HSV isolated after infection. Figure 3 shows that HSV titer reached a peak of 5 $\log_{10}$ $TCID_{50}$ (tissue culture infectious dose at 50% of the cultures) 4 days after infection. On the eighth day no virus could be isolated. Similar kinetics were observed when ALA was administered immediately after infection (Fig. 4B). However, when ALA administration was followed by light exposure no HSV could be isolated (Fig. 4A). ALA-PDT 2 days after infection had no effect on the HSV titer (Fig. 5)

Clinical Cases

Case 1. A patient who underwent kidney transplant 15 years previously presented with massive *Verrucae vulgares* of the hands (Fig. 6A). ALA (20%) in cream containing 2% EDTA and 2% DMSO was applied and the affected area exposed to red light 4 hours later (120 J/cm^2). Figure 6B and C show the results 7 days (crust formation) and 1 month after treatment, respectively. A dramatic clearance of the lesions can be seen. No recurrence occurred during a 2-year follow-up.

Case 2. An AIDS patient presented with *Molluscum contagiosum* (Fig. 7A). The red fluorescence of PpIX can be clearly seen in Fig. 7B 4 hours after application of ALA. It is noteworthy that the fluorescence extends to apparently normal skin. Dramatic regression of the lesions can

be seen (Fig. 7C) 1 month after 120 J/cm^2 of red light. No exacerbation occurred during a 4-month follow-up.

DISCUSSION

Our results clearly show that ALA-PDT can inactivate intracellular HIV in vitro and HSV in vivo. The mechanism of inactivation has not been studied but is most likely mediated by endogenous PpIX that is activated by light. In addition, there is a considerable dark effect in vitro. Because ambient light could not be excluded at all times during sample processing, it is not certain to what extent this is a real dark effect of PpIX. However, the dark toxicity of PpIX at high levels is documented and is due, in part, to interference with the cytochrome P-450 system [14]. To what extent this is the case for virally-infected cells remains to be studied.

Exogenous photosensitizers can inactivate cell-free HIV as well as intracellular HIV. The latter is inactivated only when the photosensitizer can readily penetrate the plasma membrane [15]. Thus, aluminum phthalocyanine tetrasulfonate (negatively charged) and the silicon phthalocyanine Pc 5 (positively charged) are ineffective while the silicon phthalocyanine Pc 4 (neutral) is highly effective for inactivation of cell-associated HIV. Only the latter can readily be taken up by cells [16]. The target in the cell for PpIX-induced inactivation of a virus is not known. It is probably different from that of Pc 4 since the latter causes the infected U1 cells to undergo massive apoptosis [17] while ALA-PDT did not (data not shown).

The observation that ALA-PDT is effective against HSV in vivo only during the first few hours after infection may relate to the localization of the virus. Shortly after infection, the virus may be extracellular as well as actively replicating at the site of inoculation. At later times, the virus migrates through the nerves and becomes latent. Treatment with ALA-PDT at this time has no effect on the clinical symptoms or on virus titer. In the presence of serum PpIX is transported out of the cell [8] and could serve as a sensitizer for the photodestruction of cell-free viruses. Although this point has not been addressed specifically in our study, the complete elimination of HSV in vivo by ALA-PDT shortly after infection (Fig. 4) suggests that such a mechanism may operate.

The response of the two clinical cases was dramatic, and in view of the experimental animal data, somewhat unexpected, since both presented a long time after infection. However, the latency of HSV is quite different from that of the papilloma and pox viruses. The latter two represent a persistent infection and are not truly dormant as HSV is. In addition, the different modes of application (i.p. vs. topical) preclude a direct comparison. Interestingly, in clinical Case 2 the fluorescence of PpIX extended to apparently normal skin. A likely explanation may be that this area of skin is virally infected without clinical manifestations.

For practical purposes, the clinical response is important because the treatment of refractory *verrucae vulgares* is often frustrating and a previous study of ALA-PDT resulted in five failures out of six patients [18]. Our success may be related to two improvements we have made for clinical ALA-PDT. These are the addition of DMSO to enhance ALA penetration into the skin and the use of EDTA as an iron chelator to increase the transient accumulation of endogenous PpIX [19,20]. Desferal is a more specific iron chelator than EDTA and is quite effective in enhancing PpIX accumulation in virally infected cells (Fig. 1). However, because of our previous experience with EDTA in both experimental animals [19] and clinically [20] we chose to include it also in this study. However, more extensive clinical studies are needed to demonstrate the effectiveness of this procedure for viral infections. It should be pointed out that for some indications, such as *condylomata acuminata* (genital warts), ALA-PDT with no additives can be quite effective [21].

In conclusion, addition of ALA to virally infected cells can result in dramatic accumulation of PpIX in the cells under appropriate conditions. Subsequent exposure to red light can result in drastic reduction in virus infectivity. When properly done, ALA-PDT can clear viral lesions in both experimental animals and human patients.

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